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RESEARCH PAPER

A positive allosteric modulator of α7 nAChRs augments neuroprotective effects of endogenous nicotinic agonists in cerebral ischaemia

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BACKGROUND AND PURPOSE

Activation of α7 nicotinic acetylcholine receptors (nAChRs) can be neuroprotective. However, endogenous choline and ACh have not been regarded as potent neuroprotective agents because physiological levels of choline/ACh do not produce neuroprotective levels of α7 activation. This limitation may be overcome by the use of type-II positive allosteric modulators (PAMs-II) of α7 nAChRs, such as 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea (PNU-120596). This proof-of-concept study presents a novel neuroprotective paradigm that converts endogenous choline/ACh into potent neuroprotective agents in cerebral ischaemia by inhibiting α7 nAChR desensitization using PNU-120596.

EXPERIMENTAL APPROACH

An electrophysiological *ex vivo* cell injury assay (to quantify the susceptibility of hippocampal neurons to acute injury by complete oxygen and glucose deprivation; COGD) and an *in vivo* middle cerebral artery occlusion model of ischaemia were used in rats.

KEY RESULTS

Choline (20–200 μM) in the presence, but not absence of 1 μM PNU-120596 significantly delayed anoxic depolarization/injury of hippocampal CA1 pyramidal neurons, but not CA1 stratum radiatum interneurons, subjected to COGD in acute hippocampal slices and these effects were blocked by 20 nM methyllycaconitine, a selective α7 antagonist, thus, activation of α7 nAChRs was required. PNU-120596 alone was ineffective *ex vivo*. In *in vivo* experiments, both pre- and post-ischaemia treatments with PNU-120596 (30 mg·kg^{-1}, s.c. and 1 mg·kg^{-1}, i.v., respectively) significantly reduced the cortical/subcortical infarct volume caused by transient focal cerebral ischaemia. PNU-120596 (1 mg·kg^{-1}, i.v., 30 min post-ischaemia) remained neuroprotective in rats subjected to a choline-deficient diet for 14 days prior to experiments.

CONCLUSIONS AND IMPLICATIONS

PNU-120596 and possibly other PAMs-II significantly improved neuronal survival in cerebral ischaemia by augmenting neuroprotective effects of endogenous choline/ACh.

Abbreviations

COGD, complete oxygen and glucose deprivation; GBZ, gabazine; MCAO, middle cerebral artery occlusion; MLA, methyllycaconitine; PAM-II, positive allosteric modulator; PNU-120596, 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea

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Introduction

Stroke remains the third leading cause of death in the United States and over 85% of all strokes are ischaemic strokes (Beal, 2010; Roger *et al.*, 2012). Over one third of stroke victims do not survive the incidence of stroke; while over 90% of stroke survivors suffer from permanent neurological deficits (Jorgensen *et al.*, 1999; Roger *et al.*, 2012). Clinical management of neuronal damage resulting from ischaemic stroke generally involves only palliative treatments. Currently, the only Food and Drug Administration-approved drug therapy for ischaemic stroke involves the i.v. use of tissue plasminogen activator (tPA) to dissolve clots (Furlan, 2002). This strategy appears to be effective in ischaemic stroke, but only within the first 3 h after the onset of ischaemic stroke (Brott and Bogousslavsky, 2000; Adams *et al.*, 2007). These strict limitations reduce the percent of stroke patients eligible for tPA to as low as ~2% (Katzan *et al.*, 2000). Although in the last two decades, substantial efforts have been invested in developing neuroprotective medicine, these efforts have not resulted in clinically efficacious therapies for ischaemic stroke (Richard Green *et al.*, 2003). These failures highlight the need for development of new concepts, therapeutic ideas and approaches for neuronal protection and prevention of neuronal damage secondary to ischaemia. In this search, strategies that are based on recruiting and activating endogenous pathways receive special attention as these approaches are expected to be highly efficacious and cause fewer adverse effects than approaches that utilize exogenous agents (Lo, 2008; Uteshev, 2012a). Development of these drug therapies would be expected to specifically benefit individuals from high-risk groups for ischaemic stroke (Roger *et al.*, 2012).

There is a growing pool of supportive evidence linking activation of α7 nicotinic acetylcholine receptors (nAChRs) to enhanced neuronal survival and resistance to ischaemia and other insults (Meyer *et al.*, 1998; Shimohama *et al.*, 1998; Li *et al.*, 1999; Rosa *et al.*, 2006; Guseva *et al.*, 2008), as well as improved cognitive performance in patients and animal models of schizophrenia (Olincy *et al.*, 2006; Olincy and Stevens, 2007), dementia (Kem, 2000; Ren *et al.*, 2007; Bitner *et al.*, 2010) and traumatic brain injury (Guseva *et al.*, 2008). This study is a proof of concept that introduces a novel approach to reduction of neuronal injury secondary to ischaemia by augmenting neuroprotective effects of endogenous choline/ACh. Choline is a full selective agonist of α7 nAChRs (Papke *et al.*, 1996; Alkondon *et al.*, 1997), a ubiquitous cell membrane-building material and a precursor metabolite of ACh. However, at physiological levels (i.e. <20 μM) and pathophysiological levels (i.e. >20 μM) expected in the penumbra of brain injury (Klein *et al.*, 1998; Sarter and Parikh, 2005), choline alone is ineffective as an α7 nAChR agonist because of its low potency for α7 activation (EC_{50}~0.5 mM; Papke and Papke, 2002) and tendency to induce α7 desensitization (IC_{50}~40 μM; Uteshev *et al.*, 2003). As a result, choline has not previously garnered much attention as a potential neuroprotective agent. These limitations may be overcome by the use of a previously reported novel class of compounds, type-II positive allosteric modulators (PAMs-II) of α7 nAChRs, such as 1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea (PNU12596) (Hurst *et al.*, 2005; Gusev and Uteshev, 2010; Kalappa *et al.*, 2010; Uteshev, 2012a,b). PNU-120596 does not activate α7 nAChRs, but it inhibits desensitization and enhances activation of α7 nAChRs by nicotinic agonists, including choline and ACh (Hurst *et al.*, 2005; Gusev and Uteshev, 2010; Kalappa *et al.*, 2010). In the presence of PNU-120596, endogenous levels of choline/ACh may become neuroprotective by producing a weak persistent and tunable modality of α7 nAChR activation (Gusev and Uteshev, 2010; Kalappa *et al.*, 2010; Sitzia *et al.*, 2011; Uteshev, 2012b). However, excessive activation of α7 nAChRs can be neurotoxic (Del Barrio *et al.*, 2011; Dinklo *et al.*, 2011) because of the high permeability of α7 nAChR-mediated ion channels to Ca^{2+} ions (Vernino *et al.*, 1992; Castro and Albuquerque, 1995; Fucile, 2004; Uteshev, 2010).

This study tests the hypothesis that PNU-120596 converts physiological/endogenous choline/ACh into potent neuroprotective agents in both *ex vivo* and *in vivo* experimental models of ischaemic stroke. To conduct these tests, an electrophysiological neuronal injury assay was developed and applied to hippocampal CA1 pyramidal neurons and CA1 interneurons in whole-cell current-clamp patch-clamp experiments in rat acute hippocampal slices subjected to complete oxygen and glucose deprivation (COGD). The COGD protocol was used in acute hippocampal slices (*ex vivo*) to model conditions that are likely present in the core (as opposed to the penumbra) of the ischaemic injury where neuronal injury and death can be delayed, but not eliminated (Lo, 2008). The effects of s.c. and i.v. administration of PNU-120596 on the ischaemic infarct volume within cortical and subcortical brain regions of the forebrain were evaluated in *in vivo* experiments in rats using the middle cerebral artery occlusion (MCAO) model of stroke. Some *in vivo* experiments were done in rats that were subjected to a choline-deficient diet for 14 days prior to experimentation. The results of this study support the hypothesis that PAMs-II can recruit and activate endogenous agonists of α7 nAChRs (i.e. choline and ACh) to significantly enhance neuronal survival in cerebral ischaemia.

Materials and methods

Animals

Young adult male Sprague-Dawley (S.-D.) rats (a total of 181 animals) were used for *ex vivo* (~150 g) and *in vivo* (~280 g) experiments. The animal use was in accordance with the Guide for the Care and Use of Laboratory Animals (NIH 865-23, Bethesda, MD), and all experimental protocols were approved by the Animal Care and Use Committee of Southern Illinois University School of Medicine, Springfield, IL and the Institutional Animal Care and Use Committee of University of North Texas Health Science Center at Fort Worth, TX. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Choline-deficient diet

Male S.-D. rats (~150 g) were purchased from Charles Rivers Laboratories International, Inc. (Wilmington, MA, USA) and subjected to an *ad libitum* choline-deficient diet (TD.88052; Harlan Laboratories, Inc., Indianapolis, IN, USA) for 14 days upon arrival to the UNTHSC. Water was given *ad libitum*.

Animal's weight was measured every 3 days. After 14 days, animals whose weight exceeded 260 g were selected for experimentation. Underweight animals were used for other projects. The average weight gain of choline-deficient rats (~5.5% per day) was comparable with that reported for choline-sufficient rats of the same age (~5.8% per day) (http://www.criver.com/SiteCollectionDocuments/rm_rm_c_sprague_dawley_rats.pdf).

Tissue preparation

Coronal whole-brain slices of 250–300 μm thickness containing hippocampal CA1 region were cut in a sucrose-rich solution at 3°C using Vibratome-1000 + slicer (Leica Microsystems, Wetzlar, Germany) upon euthanizing via rapid decapitation. The sucrose-rich solution was of the following composition (in mM): sucrose 250, KCl 3, NaH_2PO_4 1.23, $MgCl_2$ 5, $CaCl_2$ 0.5, $NaHCO_3$ 26, glucose 10 (pH = 7.4, when bubbled with carbogen: 95% O_2 and 5% CO_2). Slices were then transferred to a temporary storage chamber where they were maintained for ~30 min at 30°C in an oxygenated artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl 125, KCl 3, NaH_2PO_4 1.23, $MgCl_2$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10 (pH = 7.4, when bubbled with carbogen). Slices were then transferred to the long-term storage chamber and maintained at room temperature for up to 6 h bubbled with carbogen.

Patch-clamp electrophysiology in acute brain slices

The recording chamber (volume 2 mL) was perfused with oxygenated ACSF at a rate of 1 mL·min^{-1} using a perfusion pump (Model73160-20, Cole-Parmer, Vernon Hills, IL, USA). Hippocampal CA1 pyramidal neurons and CA1 stratum radiatum interneurons in brain slices were selected on the basis of their morphology and location within the slice as reported previously (Kalappa *et al.*, 2010). Neurons were visually selected for electrophysiological recordings using an Olympus BX-51WI microscope (Olympus Inc., Center Valley, PA, USA). Conventional whole-cell patch-clamp electrophysiological recordings were conducted using a MultiClamp-700B amplifier equipped with Digidata-1440A A/D converter (Molecular Devices, Sunnyvale, CA, USA). The membrane resting potential was continuously recorded in the current-clamp configuration. Data were filtered at 2–4 kHz, sampled at 10 kHz and stored on a personal computer. Patch pipettes were pulled using a Sutter P-97 horizontal puller (Sutter Instruments, Novato, CA, USA). The pipette resistance was 4–6 MΩ when filled with the internal solution. The access resistances (<30 MΩ) were not compensated. The extracellular recording solution was identical to ACSF used for the tissue preparation. The patch electrode solution contained (in mM): K-gluconate 140, NaCl 1, $MgCl_2$ 2, Mg-ATP 2, Na-GTP 0.3, HEPES 10, KOH 0.42 (pH 7.4). Membrane voltages were not corrected for the liquid junction potential: V_{LJ}(K-gluconate) = 16.2 mV. All *ex vivo* experiments were conducted at 30–32°C.

Oxygen-glucose deprivation and pretreatment of acute hippocampal slices with PNU-120596 and choline

CA1 pyramidal neurons and CA1 interneurons in acute hippocampal slices subjected to COGD (i.e. no reperfusion) were used in all experiments. COGD was achieved by a continuous perfusion of slices with an oxygen-glucose deprived ACSF (i.e. COGD-ACSF) containing 10 mM sucrose instead of 10 mM glucose and bubbled with 95% N_2 + 5% CO_2 instead of carbogen (i.e. 95% O_2 + 5% CO_2). Hippocampal slices were prepared as described above and then, randomly separated into two groups, 'control' or 'treatment', and stored for ~40 min in a chamber perfused with a standard ACSF bubbled with carbogen at room temperature. 'Treatment' slices were then transferred to 'treatment' chamber and subjected to a specific treatment for 3 h: for example, 20 μM choline +1 μM PNU-120596. 'Control' slices were transferred to 'control' chamber and incubated in standard ACSF for the same duration. After treatments, slices were transferred into the recording chamber where they remained perfused in the corresponding solutions during recordings. To initiate COGD, standard ACSF was replaced with COGD-ACSF by switching solutions that enter the recording chamber. The solution exchange delay time (i.e. time necessary to replace the system's 'dead' volume after a solution switch) was estimated using high concentrations of a food dye (203 ± 13 s, n = 3) and not subtracted from data.

Electrophysiological cell injury assay

Hippocampal CA1 pyramidal neurons and CA1 stratum radiatum interneurons in acute coronal slices subjected to COGD were used in all experiments. In total, 107 hippocampal CA1 pyramidal neurons and 16 stratum radiatum interneurons were used in this study. Each hippocampal slice was used only for one experiment utilizing COGD and only one neuron was tested in each slice. Each treatment with nicotinic agents had its own control data. This was achieved by using slices obtained from the same animal and preparation for both control (i.e. COGD only) and treatment (i.e. COGD + PNU + choline) sets of experiments to ensure the presence of control data sets for each animal and preparation.

An electrophysiological cell injury assay was developed and used in this study to achieve a reliable and continuous quantification of the evolution of neuronal anoxic depolarization and injury. Similar approaches have been used previously to study the effects of OGD on the mechanisms of cell death (Tanaka *et al.*, 1997; Dijkhuizen *et al.*, 1999; Rossi *et al.*, 2000). The effects of choline + PNU-120596 pretreatment on the status of neuronal health or injury were determined by continuous recordings of the neuronal membrane potential before and during the insult (i.e. COGD) or treatment (i.e. choline + PNU-120596) using the conventional current-clamp whole-cell patch-clamp electrophysiology: healthy cells were defined by the normal resting membrane potential (i.e. near-60 mV), while injured cells were defined by depolarized membrane potentials (i.e. more positive than-50 mV) and dead cells were assumed to have the resting membrane potential near 0 mV. Therefore, the evolution of acute neuronal depolarization/injury by COGD was monitored in real time and injury curves were built and used for analysis. To build averaged injury curves, data points pertaining to individual injury curves obtained under identical experimental conditions (e.g. control or treatment) were averaged point-by-point across all neurons in a given group. However, the mean T_{50} values presented in Figures 2–3 were the averages of T_{50} obtained from the corresponding individual injury

curves, not the T_{50} values obtained from the averaged injury curves. While the end result of OGD may be the same (i.e. neuronal depolarization/injury or death defined as a loss of the ionic gradient and the resting membrane potential), the injury curves may be different for different cell types and experimental treatments. Reperfusion of neurons defined as dead (i.e. the neuronal membrane potential was near 0 mV) with normal ACSF did not hyperpolarize the neuronal membrane potential supporting the definition. Transient or partial oxygen-glucose deprivation models where oxygen and/or glucose were briefly or only partially removed from ACSF were not investigated in this study.

Electrophysiological data analysis

The neuronal membrane potential was recorded in 10 s episodes without any delays between episodes to generate the mean membrane potential every 10 s. Thus, six means were generated every minute of recordings and the resulting injury curves were used for analysis (Figure 1). Action potentials were ignored during analysis and the membrane potential in between the neighbouring action potentials was used for analysis. For clarity, some illustrations were made using only every other means (i.e. three means per minute of recordings). The neuronal depolarization/injury was quantified by half-time to the membrane voltage 0 mV (i.e. T_{50}). Neurons not subjected to COGD maintained their resting membrane potentials relatively constant (n = 8; Figures 1A,B and 2A). By contrast, all tested neurons subjected to COGD depolarized to 0 mV as a result and thus, were defined as dead (Figures 1C–F and 2B–F). For each neuron, T_{50} was estimated as duration from the moment of solution switch (i.e. t = 0) to the point where the membrane voltage drops 50% from its initial value (i.e. at t = 0). The T_{50} was then estimated by assuming that the membrane potential between the data points just above (T_1, V_1) and just below (T_2, V_2) the point of 50% drop (i.e. within a maximum of <10 s of recordings) was a linear function of time: $T_{50} = T_1 + (T_2 - T_1)*(V_1 - 50)/(V_1 - V_2)$. In this analysis, V was normalized to the resting membrane potential at t = 0 to reduce interneuronal variability in the membrane resting potentials. The T_{50} obtained under control (i.e. COGD only) and treated (i.e. COGD + treatments) conditions and averaged over the corresponding sets of neurons were compared using the standard unpaired two-tailed Student's test (GraphPad Software, Inc., La Jolla, CA, USA). Data fitting was done using ProStat analysis packages (Pearl River, NY, USA). Statistical significance was defined by the P-value (i.e. $P < 0.05$). Graphs were built using GraphPad and ProStat data analysis software packages. Data were presented as means ± SEM.

MCAO and infarct volume measurement

The results obtained in *ex vivo* experiments (Figures 1–4) suggested that PNU-120596 may produce significant neuroprotection *in vivo*. To test this hypothesis, transient focal cerebral ischaemia was induced using the suture occlusion technique as previously described (Jin *et al.*, 2001). Animals (n = 64; Charles River) were anaesthetized with 4% isoflurane mixed with 67% N_2O and 29% O_2 and delivered by a mask. After a midline incision in the neck, the left external carotid artery (ECA) was carefully exposed and dissected. A 19 mm, 4-0 monofilament nylon suture was inserted from the ECA into the left internal carotid artery to occlude the origin of left middle cerebral artery. After 90 min of occlusion, the thread was removed to allow reperfusion. The ECA was ligated, and the wound was closed. Rectal temperature was maintained at ~37°C using a heating pad.

Rats were anaesthetized and decapitated 24 h after MCAO. Brains were removed and coronal sections (2 mm thickness) immersed in 2% 2,3,5-triphenyltetrazolium chloride in saline for 20 min at 37°C, then fixed for 2 h in 4% paraformaldehyde (Bederson *et al.*, 1986). Infarct area, left hemisphere area and total brain area were measured by a blinded observer using the Image J software, and areas were multiplied by the distance between sections to obtain the respective volumes. Infarct volume was calculated as a percentage of the volume of the contralateral hemisphere, as described previously (Swanson *et al.*, 1990). Statistical significance was defined by the P-value (i.e. $P < 0.05$) using the two-tailed unpaired Student's t-test.

MS analysis of PNU-120596 amounts in brain/blood samples

Animals were anaesthetized with 4% isoflurane mixed with 67% N_2O and 29% O_2 (delivered by a mask) and perfused with ACSF. Samples of blood and hippocampal, frontal cortical and striatal brain tissues were collected, flash-frozen using liquid nitrogen, stored at -80°C at UNTHSC (Fort Worth, TX, USA) and shipped on dry ice overnight to Carbon Dynamics Institute, LLC (Springfield, IL, USA) for MS analyses. Subsamples were macerated with methanol for 3 min and the resultant slurry was centrifuged at 4°C for 15 min at 15 000× g. Blood samples were centrifuged at 4°C for 15 min at 19 000× g. The resultant supernatant for both preparations was further purified using a mixed-mode polymeric exchange solid phase extraction (SPE). The eluate was lyophilized and reconstituted in the initial mobile phase. PNU-120596 was analysed by a one microliter injection of each sample aliquot into a Waters 2795 Alliance equipped with a XBridge BEH130 C_{18} 2.1 mm × 150 mm, 3.5 µm particulate column (110 Å) at a flow rate of 0.150 mL·min^{-1}; a binary mobile phase gradient at 50:50 (Δ2.5%) A : B in 20 min (Mobil phase A = 0.01% formic acid; Mobil phase B = acetonitrile) until proper peak shape, separation and reduction of interferences were obtained. Waters Quattro Ultima triple stage quadrupole mass spectrometer equipped with an electrospray ionization source was calibrated with methionine-arginine-phenylalanine-alanine peptide for both the single and double charge state (m/z 524.2 and 262.6, respectively) to provide a 0.1 amu mass accuracy for each $[M + H]^+$ parent ion (m_p^+). The m_p^+ ion 312 from Q1 was passed through a collision chamber (Q2), operating in radio-frequency-only mode (23 V), and focused for subsequent ionization as a consequence of the collision of the rapidly moving m_p^+ with an inert gas (Ar) at ~1.8 mTorr to produce the unique product fragmentation spectrum that was subsequently scanned through a third mass filter (Q3) where selected daughter ions (m_d^+) were collected in multiple resonance monitoring (transitions 312 > 188 173.99).

Drugs

PNU-120596 was obtained from the National Institute of Drug Addiction through the Research Resources Drug Supply

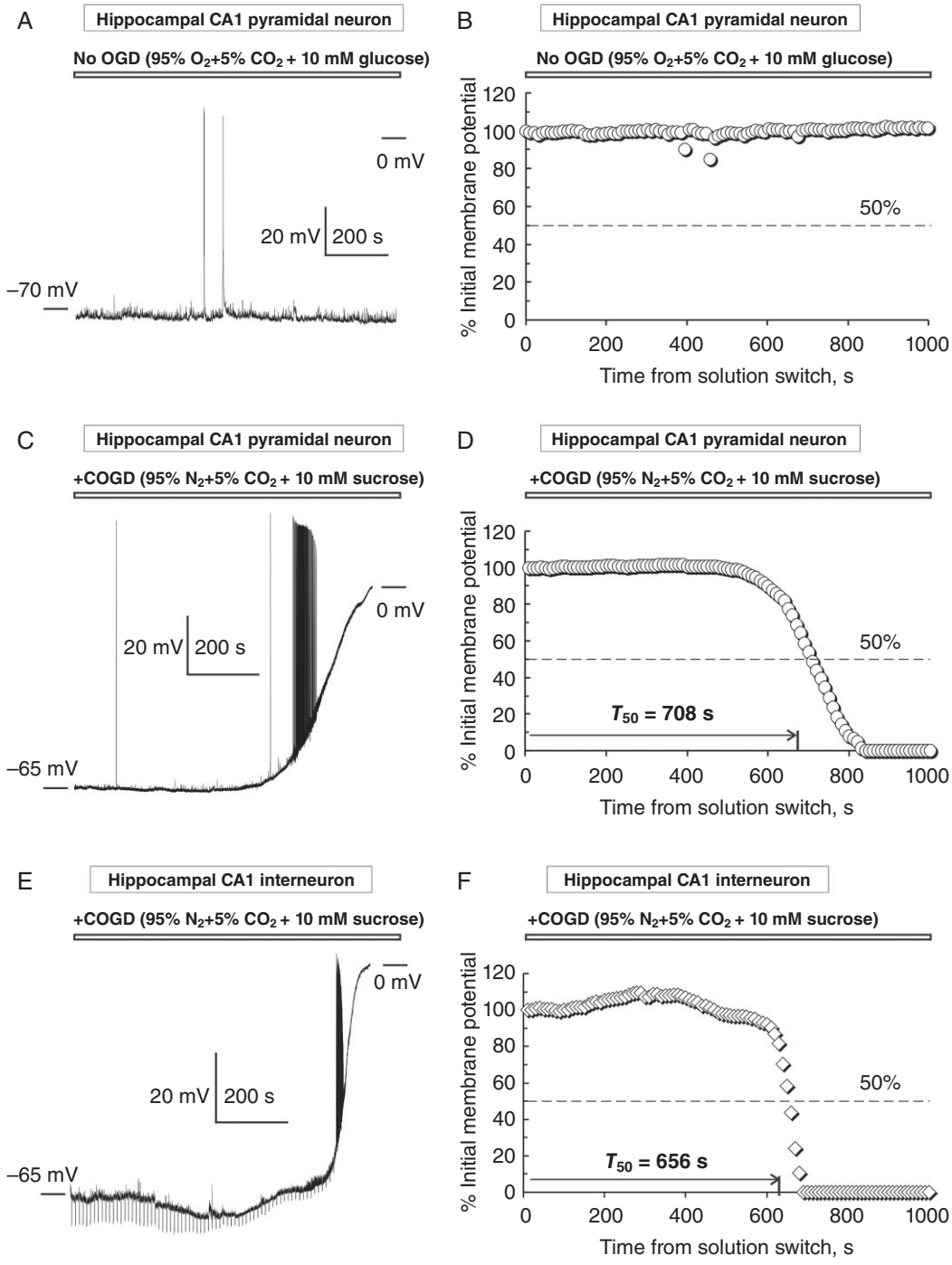

Figure 1

Examples of COGD-induced injury curves. Typical examples of the raw (A, C and E) and the normalized (B, D and F) injury curves recorded and built for two representative hippocampal CA1 pyramidal neurons (no OGD, A–B and COGD, C–D) and a hippocampal CA1 interneuron (E–F) in *ex vivo* experiments in acute hippocampal slices. As expected, no injury was detected in the absence of OGD (A–B; $n = 5$). Each data point in the injury curves (i.e. D and F) indicates the mean normalized membrane potential calculated over a 10 s episode as a function of time from solution switch (from ACSF to COGD-ACSF). There was no solution switch in experiments without OGD (i.e. A–B). Each injury curve (i.e. C–D and E–F) is quantified by the value of T_{50} indicating the half-time to 0 mV. The T_{50} was not defined in experiments without OGD (i.e. A–B). The dashed lines in D) and F) indicate 50% drop from the initial membrane potential. For clarity of illustrations, only every other data point is shown in D and F. All data points were used in data analysis to determine T_{50}. Neurons used in these examples were obtained from different preparations and investigated on different experimental days. Regular downward voltage deviations in E were hyperpolarizing (–20 pA) steps used in some recordings to determine the neuronal input resistance. Depolarization-induced action potentials clearly seen in the raw recordings (C and E) were ignored during analysis and thus, are not represented in the normalized injury curves (D and F). Two strong spontaneous bursts of action potentials in A are represented as small downward deviations in B.

Program and purchased from Tocris Bioscience (Ellisville, MO, USA) and Selleck Chemicals (Houston, TX, USA). Methyllycaconitine citrate (MLA) was purchased from Ascent Scientific Ltd. (Bristol, UK). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nomenclature of drugs and molecular targets conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011).

PNU-120596

In *ex vivo* experiments, 1 μM PNU-120596 was used. This concentration lies near the EC_{50} for potentiating effects of PNU-120596 in heterologous systems (EC_{50} ~1.5 μM; Gronlien *et al.*, 2007; Young *et al.*, 2008). Intravenous administration of 1 mg·kg^{-1} PNU-120596 has been shown to elevate the concentration of PNU-120596 in the brains of rats to similar values (~1.5 μM; Hurst *et al.*, 2005). In *in vivo* experiments, 1 mg·kg^{-1} (*in vivo*, i.v.). and 30 mg·kg^{-1} (*in vivo*, s.c.) were used. Similar doses have been used in other studies (Hurst *et al.*, 2005; McLean *et al.*, 2011). PNU-120596 is a highly lipophilic compound and thus, can be trapped by the tissue near the s.c. injection site. Therefore, the dose used for s.c. injections was chosen higher than that for i.v. injections to compensate for that possibility. To make a 50 mM stock solution (maximal achievable concentration is ~200 mM), PNU-120596 was dissolved in dimethyl sulfoxide (DMSO). However, the amount of DMSO injected in each animal did not exceed 2 mL·kg^{-1}. The appropriate amounts of 50 mM stock solution PNU + DMSO or DMSO alone (i.e. control) were injected as a bolus s.c. (30 mg·kg^{-1}) or i.v. (1 mg·kg^{-1}).

Results

This study used *ex vivo* (i.e. COGD) and *in vivo* (i.e. MCAO) experimental approaches to test the hypothesis that PNU-120596, a type-II PAM of α7 nAChRs, converts physiological/endogenous choline/ACh into potent neuroprotective agents via activation of α7 nAChRs. All *ex vivo* patch-clamp experiments were conducted at 30–32°C.

Injury curves and quantification of the evolution of COGD-induced acute neuronal injury

Subjecting acute hippocampal slices to COGD resulted in a permanent loss of the ionic gradient and depolarization of the neuronal membrane potential leading to neuronal injury and death. The stages of neuronal injury were quantified by the time-dependent decrease in the neuronal membrane potential (i.e. anoxic depolarization), a key parameter defining the status of neuronal health and the evolution of neuronal injury (i.e. injury curves). Figure 1 illustrates typical examples of the raw (recorded in current-clamp) and the normalized individual injury curves obtained from two hippocampal CA1 pyramidal neurons subjected to either control (no COGD, no pretreatment with choline + PNU-120596, Figure 1A,B) or COGD conditions (COGD, but no pretreatment with choline + PNU-120596, Figure 1C,D) and one hippocampal CA1 stratum radiatum interneuron subjected to COGD without pretreatment with choline + PNU-120596 (Figure 1E,F). As expected, neuronal depolarization/injury did not occur in the absence of COGD (Figure 1A,B) and thus, the value of half-time to 0 mV (i.e. T_{50}, see Methods) was not defined. Similar results were obtained in five control experiments and three preparations. By contrast, neurons illustrated in Figure 1C,E were subjected to COGD and exhibited injury curves with the following T_{50} values: T_{50} (pyramidal) = 707.5 s (Figure 1D) and T_{50} (inter) = 655.7 s (Figure 1F). These experiments were conducted using neurons obtained from different preparations.

Pretreatment with 20 μM choline + 1 μM PNU-120596

Two groups of acute hippocampal slices obtained from the same animal and preparation were pretreated for 3 h either in standard ACSF (i.e. control group) or ACSF containing 20 μM choline + 1 μM PNU-120596 (i.e. treatment group). The T_{50} estimated from individual injury curves were then averaged for each neuronal sub-type and experimental condition and the mean values were compared across the corresponding groups (unpaired, two-tailed Student test). Figure 2A–D illustrates averaged injury curves built for four sets of controls and treatments: (i) No OGD after pretreatment with 20 μM choline + 1 μM PNU-120596 (n = 3; Figure 2A; the error bars are invisible); (ii) control (COGD, no pretreatment, open circles, Figure 2B) versus pretreatment of CA1 pyramidal neurons with 20 μM choline + 1 μM PNU-120596 (closed circles, Figure 2B); (iii) control (COGD, no pretreatment, open circles, Figure 2C) versus pretreatment of CA1 pyramidal neurons with 20 μM choline + 1 μM PNU-120596 + 20 nM MLA (closed circles, Figure 2C) and (iv) control (COGD, no pretreatment, open circles, Figure 2D) versus pretreatment of CA1 interneurons with 20 μM choline + 1 μM PNU-120596 (closed circles, Figure 2D).

The results of analysis of these experiments (unpaired, two-tailed Student's *t*-test) were summarized in Table 1 and demonstrated that pretreatment of acute hippocampal slices with 20 μM choline + 1 μM PNU-120596 significantly delayed the COGD-induced acute injury and the corresponding depolarization of hippocampal CA1 pyramidal neurons (Figure 2B), but not CA1 interneurons (Figure 2D). However, these beneficial effects of 20 μM choline + 1 μM PNU-120596 were lost when 20 nM MLA was present in the ACSF during pretreatment and recordings (Figure 2C) indicating that activation of α7 nAChRs was required for the effects of choline + PNU-120596.

Pretreatment of CA1 pyramidal neurons with 20 μM choline alone or 1 μM PNU-120596 alone

When applied alone, 20 μM choline or 1 μM PNU-120596 do not activate α7 nAChRs (Gusev and Uteshev, 2010; Kalappa *et al.*, 2010). Therefore, to test the hypothesis that neuroprotection by choline + PNU-120596 requires α7 nAChR activation, acute hippocampal slices were pretreated for 3 h in 20 μM choline alone or 1 μM PNU-120596 alone. As expected, choline alone (closed circles; Figure 2E) and PNU-120596 alone (closed circles; Figure 2F) did not significantly delay neuronal depolarization/injury (Table 2) when compared to control conditions (open circles; Figure 2E,F), as evidenced by insignificant differences in both T_{50} and T_{80}

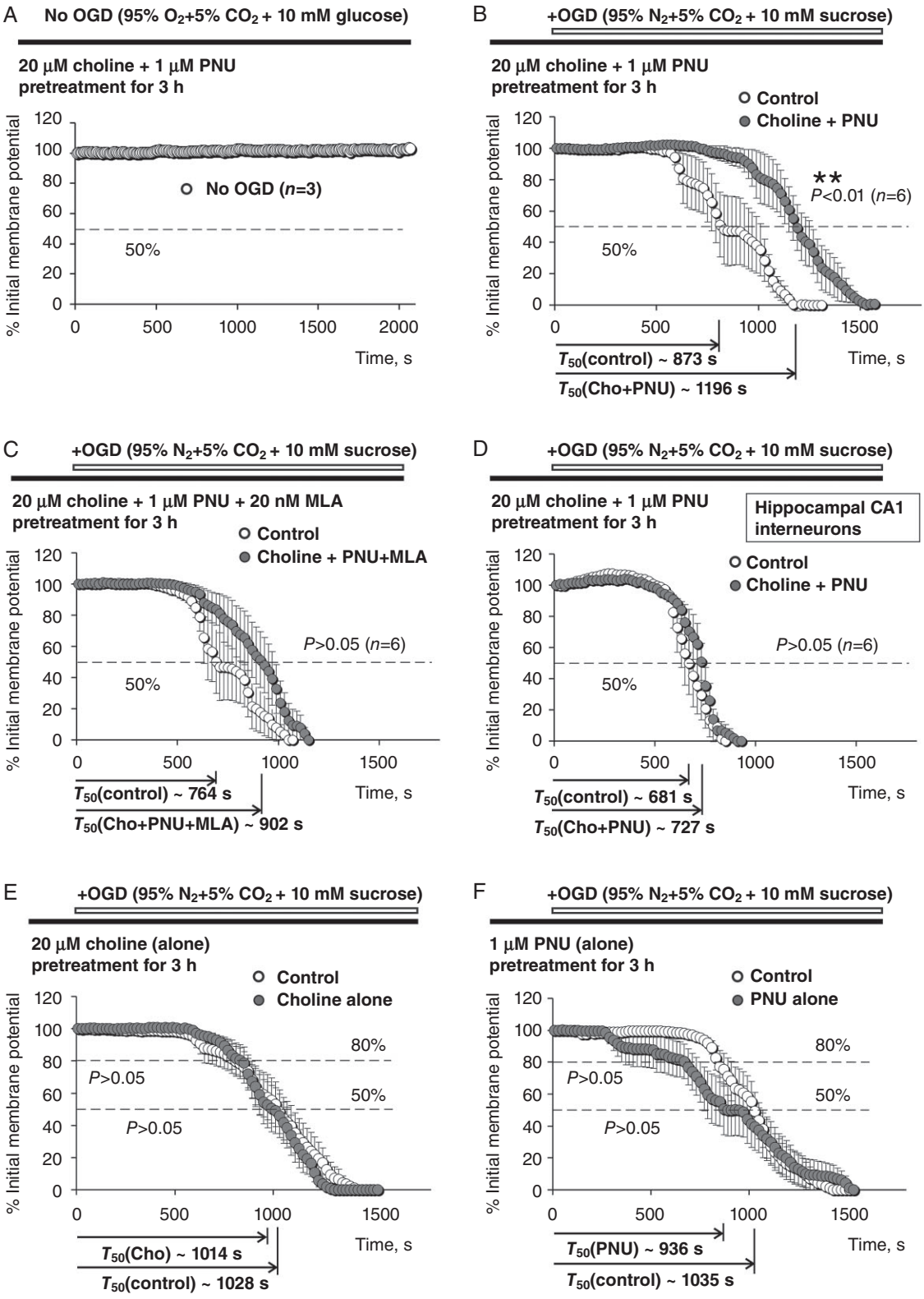
A
No OGD (95% O2+5% CO2 + 10 mM glucose)
20 μM choline + 1 μM PNU
pretreatment for 3 h
% Initial membrane potential
No OGD (n=3)
50%
Time, s
B
+OGD (95% N2+5% CO2 + 10 mM sucrose)
20 μM choline + 1 μM PNU
pretreatment for 3 h
Control
Choline + PNU
**
P<0.01 (n=6)
50%
T50(control) ~ 873 s
T50(Cho+PNU) ~ 1196 s
C
+OGD (95% N2+5% CO2 + 10 mM sucrose)
20 μM choline + 1 μM PNU + 20 nM MLA
pretreatment for 3 h
Control
Choline + PNU+MLA
P>0.05 (n=6)
50%
T50(control) ~ 764 s
T50(Cho+PNU+MLA) ~ 902 s
D
+OGD (95% N2+5% CO2 + 10 mM sucrose)
20 μM choline + 1 μM PNU
pretreatment for 3 h
Hippocampal CA1 interneurons
Control
Choline + PNU
P>0.05 (n=6)
50%
T50(control) ~ 681 s
T50(Cho+PNU) ~ 727 s
E
+OGD (95% N2+5% CO2 + 10 mM sucrose)
20 μM choline (alone)
pretreatment for 3 h
Control
Choline alone
80%
50%
P>0.05
P>0.05
T50(Cho) ~ 1014 s
T50(control) ~ 1028 s
F
+OGD (95% N2+5% CO2 + 10 mM sucrose)
1 μM PNU (alone)
pretreatment for 3 h
Control
PNU alone
80%
50%
P>0.05
P>0.05
T50(PNU) ~ 936 s
T50(control) ~ 1035 s

Figure 2

Pretreatment with 20 μM choline and/or 1 μM PNU-120596. Results of *ex vivo* experiments that employed pretreatment (3 h) of acute hippocampal slices with 20 μM choline and/or 1 μM PNU-120596. Averaged injury curves were built for four sets of controls and treatments: (A) no OGD, but after 3 h of pretreatment in 20 μM choline + 1 μM PNU-120596 ($n = 3$); (B) control (COGD, no pretreatment, open circles) versus COGD + pretreatment of CA1 pyramidal neurons with 20 μM choline + 1 μM PNU-120596 (closed circles); (C) control (COGD, no pretreatment, open circles) versus COGD + pretreatment of CA1 pyramidal neurons with 20 μM choline + 1 μM PNU-120596 + 20 nM MLA (closed circles); and (D) control (COGD, no pretreatment, open circles) versus COGD + pretreatment of CA1 interneurons with 20 μM choline + 1 μM PNU-120596 (closed circles). As expected, no injury was detected in the absence of OGD (A). Pretreatment with 20 μM choline + 1 μM PNU-120596 significantly delayed injury of hippocampal CA1 pyramidal neurons ($P = 0.0097$, $n = 6$) (B), but not CA1 interneurons (D) to COGD ($P = 0.2936$, $n = 6$). These effects were eliminated by 20 nM MLA ($P = 0.1841$, $n = 6$), a selective α7 nAChR antagonist, supporting the requirement for α7 nAChR activation (C). (E–F) The results of *ex vivo* experiments that employed 3 h pretreatment of acute hippocampal slices with 20 μM choline alone (E) or 1 μM PNU-120596 alone (F). The analysis and illustration were conducted and built as described for other panels, except that in addition to T_{50}, T_{80} was also used to compare the early phase of injury curves under control (open circles) and pretreated (closed circles) conditions. Pretreatment with 20 μM choline alone ($P = 0.8690$, $n = 10$–11) (E) or 1 μM PNU-120596 alone ($P = 0.4045$, $n = 9$–11) (F) did not significantly delay depolarization/injury of hippocampal CA1 pyramidal neurons induced by COGD supporting the requirement for α7 nAChR activation and synergistic action of choline and PNU-120596. For illustrations and to build the averaged injury curves, data points pertaining to individual injury curves obtained under identical experimental conditions (e.g. control or treatments) were averaged point-by-point across all relevant neurons. For analysis, the T_{50} values obtained from the corresponding individual injury curves (e.g. Figure 1) were averaged to determine the mean T_{50}. For clarity of illustrations, only every other data point was shown in B-D). All data points were shown in A, E–F) However, all data points were always used in data analysis to determine the mean T_{50}. The unpaired two-tailed Student's *t*-test was used for analysis (Tables 1–2).

Table 1

Choline + PNU-120596 significantly delay COGD-induced depolarization/injury of hippocampal CA1 pyramidal neurons, but not CA1 stratum radiatum interneurons and activation of α7 nAChRs is required

	Hippocampal CA1 pyramidal neurons				Hippocampal CA1 interneurons	
	Control	20 μM Cho + 1 μM PNU	Control	20 μM Cho + 1 μM PNU + 20 nM MLA	Control	20 μM Cho + 1 μM PNU
T_{50} (s) (#cells)	**873.4 ± 76.6** (6)	**1195.8 ± 65.9** (6)	764.4 ± 63.8 (6)	902.0 ± 72.3 (6)	680.7 ± 36.0 (6)	726.6 ± 20.4 (6)
P-value	**0.0097**		0.1841		0.2936	

Pretreatment with 20 μM choline + 1 μM PNU-120596 significantly delays COGD-induced depolarization/injury of hippocampal CA1 pyramidal neurons, but not CA1 stratum radiatum interneurons, and these beneficial effects are eliminated by 20 nM MLA, a selective α7 nAChR antagonist. Significant differences are shown in bold. Each experimental condition is presented in a separate column and has its own independent control.

(Table 2). The T_{80} was used to test for significant differences among the earlier phases of injury curves. Therefore, activation of α7 nAChRs was absolutely required for significant neuroprotection of hippocampal CA1 pyramidal neurons subjected to COGD *ex vivo*.

Pretreatment with 200 μM choline + 1 μM PNU-120596

Although physiologically relevant concentrations of choline (i.e. 20 μM) were found to delay the COGD-induced depolarization/injury of hippocampal CA1 pyramidal neurons in the presence, but not absence, of 1 μM PNU-120596 (Figure 2B,E and Table 1), activation of α7 nAChRs by higher concentrations of choline in the presence of PNU-120596 may result in neurotoxicity (Del Barrio *et al.*, 2011; Dinklo *et al.*, 2011) due to the high permeability of α7 ion channels to Ca^{2+} ions. To evaluate the resilience of the effects of choline + PNU-120596, experiments similar to those described above were conducted using hippocampal CA1 pyramidal neurons and a 10-fold higher concentration of choline (i.e. 200 μM). At 200 μM, choline may activate α7 nAChRs in the absence of PNU-120596 (Uteshev *et al.*, 2003). Therefore, three groups of pretreatments were used (Figure 3): (i) control (ACSF only, no choline, no PNU-120596; open circles); (ii) 200 μM choline alone (no PNU-120596; closed circles); and (iii) 200 μM choline + 1 μM PNU-120596 (open triangles). The T_{50} for individual injury curves were estimated and averaged to calculate the mean T_{50} for each group.

The results of these experiments (Table 2) demonstrated that pretreatment of acute hippocampal slices with 200 μM choline alone produced neither significant benefits, nor harm ($P = 0.4766$ vs. control; open and closed circles, Figure 3); while pretreatment with 200 μM choline + 1 μM PNU-120596 still significantly delayed neuronal depolarization/injury of CA1 pyramidal neurons subjected to COGD ($P = 0.0199$ vs. control sand $P = 0.0118$ vs. 200 μM choline alone; open triangles, Figure 3). Thus, in the presence of 1 μM PNU-120596, a broad range of choline concentrations (i.e. 20–200 μM) significantly delay depolarization/injury of hippocampal CA1 pyramidal neurons subjected to COGD *ex vivo*.

Table 2
Enhanced neuroprotection of hippocampal CA1 pyramidal neurons by synergistic action of choline + PNU-120596 in COGD

	20 μM choline alone		1 μM PNU alone		200 μM choline ± 1 μM PNU		
	Control	20 μM Cho alone	Control	1 μM PNU alone	200 μM Cho alone	Control	200 μM Cho + 1 μM PNU
T_{50} (s) (# cells)	1027.8 ± 64.9 (11)	1013.9 ± 49.8 (10)	1035.3 + 50.2 (11)	935.5 + 114.2 (9)	1041.2 ± 37.5 (5)	**1080.7 ± 37.4** (5)	**1412.6 ± 108.1** (5)
P-value		0.8690		0.4045	0.4766		**0.0199**
T_{80} (s) (# cells)	933.4 ± 72.4 (11)	903.1 ± 51.5 (10)	960.7 + 51.4 (11)	790.0 + 110.7 (9)			
P-value		0.7414		0.154			

Pretreatment with 20 μM choline alone, 200 μM choline alone or 1 μM PNU-120596 alone does not significantly delay COGD-induced depolarization/injury in hippocampal CA1 pyramidal neurons supporting the requirements for α7 activation and synergistic action of choline + PNU-120596. Significant differences are shown in bold. Each experimental condition is presented in a separate column and has its own independent control.

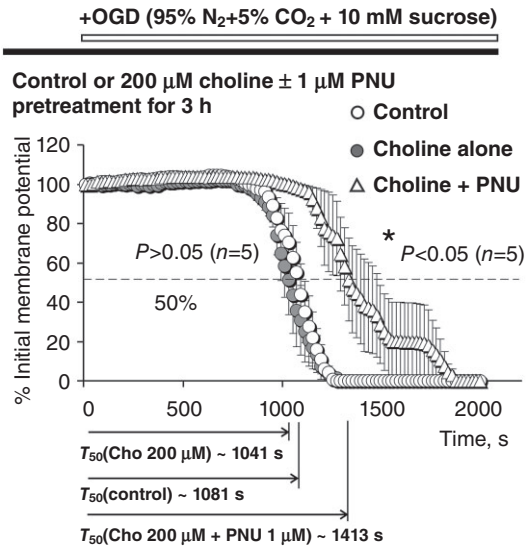

Figure 3

Pretreatment with 200 μM choline + 1 μM PNU-120596. The results of *ex vivo* experiments that employed 3 h pretreatment of acute hippocampal slices in the ACSF (open circles; control), 200 μM choline alone (closed circles) and 200 μM choline + 1 μM PNU-120596 (open triangles). The analysis and illustration were conducted and built as described for other figures. Pretreatment with 200 μM choline + 1 μM PNU-120596 (open triangles) significantly delayed COGD-induced depolarization/injury of hippocampal CA1 pyramidal neurons as compared to control (open circles; $P = 0.0199$, $n = 5$) or 200 μM choline alone (closed circles; $P = 0.0118$, $n = 5$). By contrast, pretreatment with 200 μM choline alone (closed circles) did not significantly delay injury in comparison with controls (open circles, $P = 0.4766$, $n = 5$). The unpaired two-tailed Student's *t*-test was used for analysis (Table 2).

Susceptibility of CA1 pyramidal neurons and CA1 stratum radiatum interneurons to acute injury by COGD

Injury curves obtained from subjecting CA1 pyramidal neurons and interneurons to COGD *ex vivo* in the absence of any pretreatments were compared. CA1 interneurons were found to be significantly more susceptible to COGD than CA1 pyramidal neurons because T_{50} (inter) = 680.7 ± 36.0 s (n = 6) estimated for CA1 interneurons were significantly smaller ($P = 0.0004$; $t = 3.95$; unpaired, two-tailed Student's *t*-test) than T_{50} (pyramidal) = 984.7 ± 35.1 s ($n = 27$) estimated for CA1 pyramidal neurons.

Lack of involvement of GABAergic neurotransmission

One possible mechanism that could explain the observed delay in injury of CA1 pyramidal neurons treated with choline + PNU-120596 is enhanced GABAergic inhibition of these neurons in the presence of choline + PNU-120596. CA1 pyramidal neurons are the prime target of GABAergic CA1 interneurons that express high densities of α7 nAChRs sensitive to PNU-120596 (Kalappa *et al.*, 2010). To test this hypothesis, experiments similar to those illustrated in

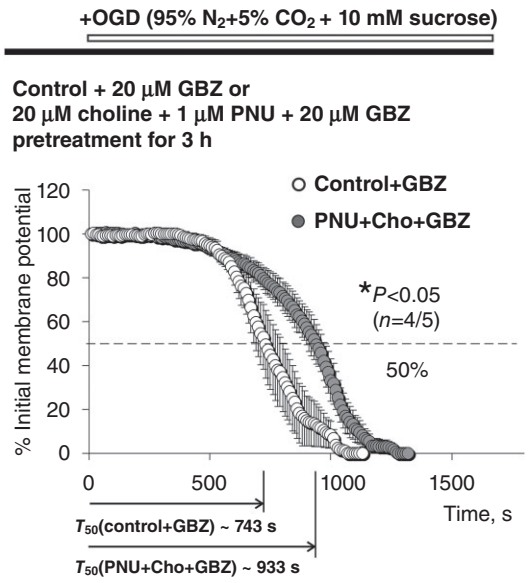

Figure 4

Lack of involvement of GABAergic neurotransmission. Experiments similar to those illustrated in Figures 2B and 3 were conducted using CA1 pyramidal neurons in the continuous presence of 20 μM GBZ in the ACSF during pretreatment of slices in 20 μM choline + 1 μM PNU-120596 and COGD. Inhibition of GABAergic neurotransmission did not alter the neuroprotective effects of choline + PNU-120596 because neuronal injury remained significantly delayed (P = 0.0198, n = 5) by choline + PNU-120596 in the presence of GBZ [T_{50} (cho + PNU + GBZ) = 933.3 ± 36.2 s, closed circles] as compared to control conditions [i.e. no pretreatment, T_{50} (control + GBZ) = 742.7 ± 55.0 s, n = 4, open circles]. The unpaired two-tailed Student's t-test was used for analysis.

Figure 2B were conducted in the continuous presence of 20 μM gabazine (GBZ), a selective $GABA_AR$ antagonist. In these experiments, GBZ was present in the ACSF during both pretreatment and COGD. Inhibition of $GABA_ARs$ did not alter the neuroprotective effects of choline + PNU-120596 on CA1 pyramidal neurons: T_{50} (control + GBZ) = 742.7 ± 55.0 s (n = 4) versus T_{50} (choline + PNU + GBZ) = 933.3 ± 36.2 s (n = 5) (P = 0.0198, Figure 4) suggesting that GABAergic inhibition is not a key mechanism of neuroprotection by choline + PNU-120596 in COGD *ex vivo*.

PNU-120596-mediated protection in an in vivo *ischaemia model*

To test the hypothesis that PNU-120596 produces significant neuroprotection *in vivo*, the MCAO model of stroke was employed in young adult S.-D. rats (~280 g). The effects of s.c. administration of PNU-120596 (30 mg·kg^{-1}) 3 h and 24 h prior to MCAO (i.e. will be referred to as '3 h pre-MCAO' and '24 h pre-MCAO', respectively) as well as i.v. administration of PNU-120596 (1 mg·kg^{-1}) 30 min after the start of MCAO (i.e. will be referred to as 'post-MCAO') on the infarct volume measured 24 h after MCAO were evaluated. The expectation was that PNU-120596 will enhance activation of α7 nAChRs by endogenous nicotinic agonists to significantly enhance neuronal resistance to MCAO and thus, significantly reduce the infarct volume caused by MCAO in both pre- and post-treatment paradigms. In each of these experiments, only the left MCA was occluded. In matching control groups of animals, only vehicle (i.e. DMSO) was injected s.c. or i.v. respectively. The results of these experiments demonstrated significant reduction in the infarct volume in 3 h pre-MCAO as well as post-MCAO treatment groups (unpaired, two-tailed, Student's t-test): P = 0.0051 (n = 4, 3 h pre-MCAO; Figure 5A–C) and P = 0.0413 (n = 5, post-MCAO; Figure 5D–F). The infarct volume was not significantly affected by PNU-120596 in the 24 h pre-MCAO treatment group (unpaired, two-tailed, Student's t-test): P = 0.5199 (n = 5, 24 h pre-MCAO; Figure 6).

PNU-120596-mediated protection in animals subjected to a choline-deficient diet

Although choline-deficient diet has been shown to significantly reduce only the plasma and not the cerebrospinal levels of choline (Klein *et al.*, 1998), the effectiveness of recruiting and activation of endogenous choline/ACh by PNU-120596 in MCAO experiments may be reduced once the animals are challenged with choline deficiency. To determine whether choline deficiency can reduce neuroprotection by PNU-120596 in MCAO experiments, S.-D. rats were subjected to a choline-deficient diet for 14 days prior to experimentation (see Methods). At the time of MCAO, the animals (>260 g) were divided into two groups: control (DMSO only) and treated (PNU-120596, 1 mg·kg^{-1}, i.v., 30 min post-MCAO). Other experimental details and data analysis were identical to those used in choline-sufficient animals (see above and Methods). In choline-deficient animals, PNU-120596 remained effective and produced significant (P = 0.0343; unpaired, two-tailed, Student's t-test) reduction in the MCAO-induced infarct volume in treated (n = 4) vs. control (n = 4) animals (Figure 7).

Evaluation of blood/brain levels of PNU-120596

To confirm the presence of PNU-120596 in the brain tissue and blood plasma, samples of blood plasma and hippocampal, frontal cortical and striatal tissues of five animals (S.-D. rats, ~280 g) subjected to a protocol utilizing 3 h pre-MCAO were evaluated using MS (see Methods). The results of MS analyses demonstrated that the amount of PNU-120596 in the hippocampus, frontal cortex and striatum collected 3 h after s.c. injections of 30 mg·kg^{-1} PNU-120596 in S.-D. rats were comparable (in ng per g of tissue): 189.5 ± 67.8 (n = 5), 157.5 ± 49.0 (n = 5) and 149.0 ± 42.7 (n = 5), respectively, and somewhat higher than the amounts of PNU-120596 remaining in the blood plasma of injected animals (in nM): 63.4 ± 19.3 (n = 5). These values are comparable to those reported previously (McLean *et al.*, 2011) as well as the concentrations (i.e. 1 μM) used in our *ex vivo* experiments (Figures 1–4). These results suggest that sub-μM to low-μM concentrations of PNU-120596 and the corresponding doses of systemically administrated PNU-120596 significantly enhance neuronal survival in ischaemic injury.

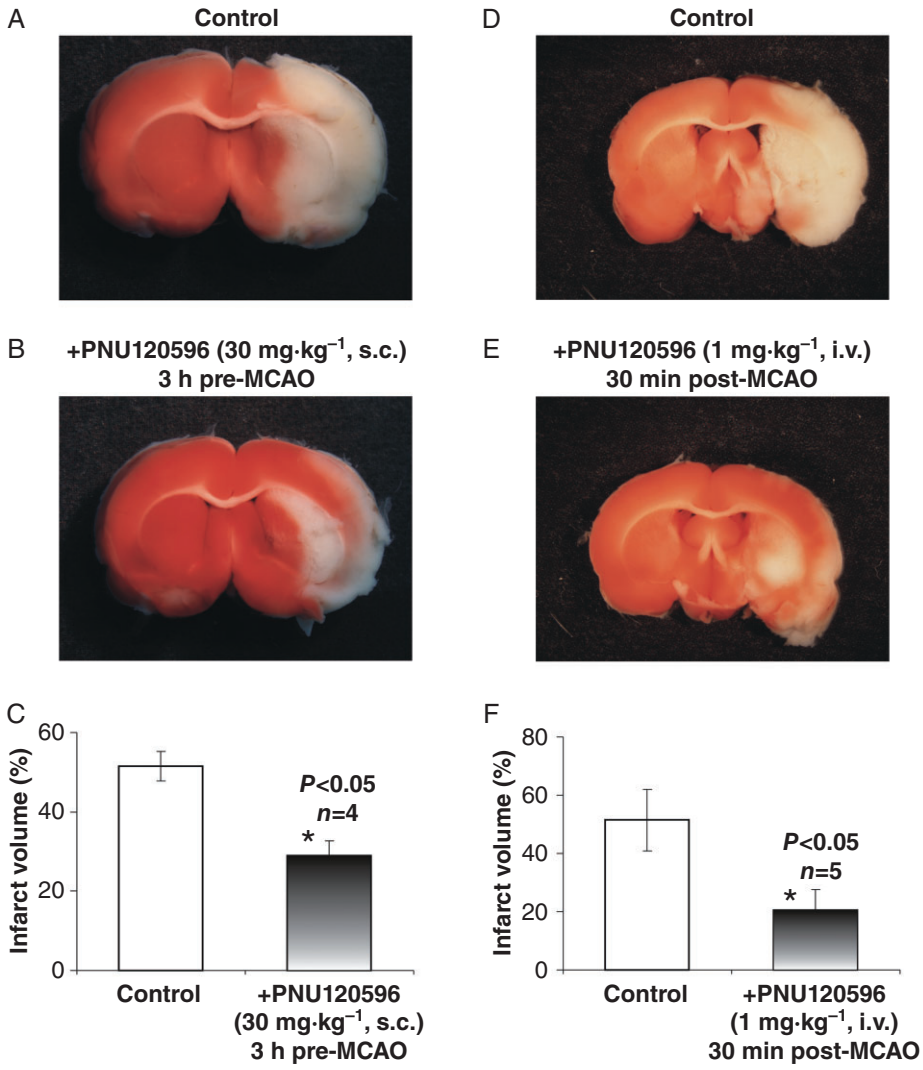

Figure 5

PNU-120596 enhances resistance of cortical/subcortical brain regions to injury induced by focal cerebral ischaemia. Focal cerebral ischaemia was induced by a transient (90 min) MCAO. Typical examples of injured whole-brain coronal sections (2 mm thickness) treated with vehicle (i.e. DMSO) only (A and D), PNU-120596 (30 mg·kg^{-1}, s.c., 3 h pre-MCAO, $n = 4$) (B) or PNU-120596 (1 mg·kg^{-1}, i.v., 30 min post-MCAO, $n = 5$) (E) respectively. Twenty-four hours after MCAO, brain sections were prepared for analysis of infarct volume within cortical and subcortical brain regions from control and treated groups. The results indicate that both 3 h pre-MCAO treatment with PNU-120596 (30 mg·kg^{-1}, s.c.) and 30 min post-MCAO treatment with PNU-120596 (1 mg·kg^{-1}, i.v.) significantly (unpaired, two-tailed, Student's *t*-test) reduced infarct volume ($P = 0.0051$, $n = 4$; C and $P = 0.0413$, $n = 5$; F respectively).

Discussion

A deficient blood supply to the whole brain, as in global ischaemia, and specific cortical and subcortical brain regions, as in focal ischaemia, may irreversibly damage brain tissues and cause a partial or complete loss of cognitive, autonomic and motor functions. Although in the last two decades, substantial efforts have been invested in developing neuroprotective medicine, these efforts have not resulted in clinically efficacious therapies for ischaemic stroke (Roger *et al.*, 2012). These failures highlight the need for development of new therapeutic concepts, approaches and drug treatments for neuronal protection and prevention of neuronal damage secondary to ischaemic stroke. The results of this proof-of-concept study support the hypothesis that PAMs-II, such as PNU-120596, can convert endogenous agonists of α7 nAChRs (i.e. choline and ACh) into potent neuroprotective agents in post-ischaemic neuronal injury in cortical and subcortical brain regions. The proposed strategy incorporates the neuroprotective capacity of α7 nAChR activation by endogenous choline/ACh enhanced by PNU-120596 (Hurst *et al.*, 2005; Gusev and Uteshev, 2010).

α7 nAChRs are commonly expressed in the brain including the hippocampus, cortex and striatum (Breese *et al.*, 1997; Whiteaker *et al.*, 1999; Quik *et al.*, 2009; Woodruff *et al.*, 2011) – brain regions that are selectively vulnerable to ischae-

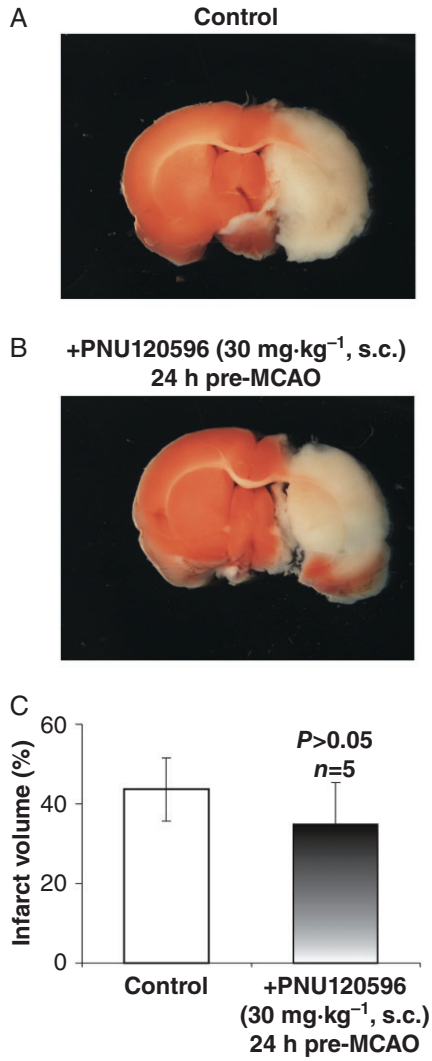

Figure 6

PNU-120596 administered s.c. 24 h prior to focal cerebral ischaemia failed to enhance resistance of cortical/subcortical brain regions to ischaemic injury. To test the hypothesis that PNU-120596 preconditions cortical and subcortical brain regions by enhancing neuronal resistance to ischaemia, PNU-120596 was injected s.c. 24 h prior to MCAO. The half-life time of PNU-120596 is ~8 h (McLean *et al.*, 2011). It was expected that PNU-120596 preconditions neurons and thus, its presence is not required at the time of ischaemic injury (i.e. 24 h after PNU-120596 injection). However, the effects of 24 h pre-MCAO s.c. treatment with PNU-120596 were found to be statistically insignificant (unpaired, two-tailed, Student's *t*-test) suggesting that the presence of PNU-120596 and thus, the enhanced activation of α7 nAChRs around the time of ischaemic injury are required for enhanced resistance to ischaemic injury.

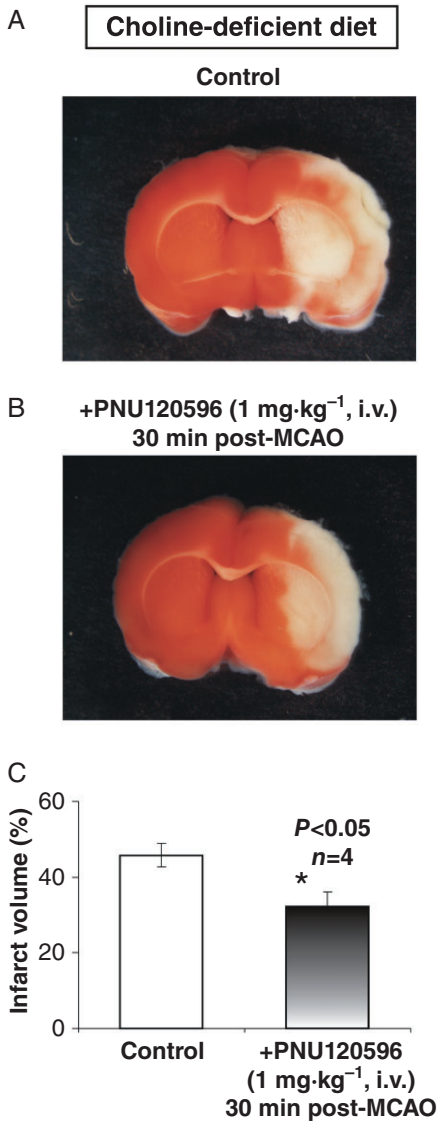

Figure 7

Neuroprotective effects of PNU-120596 remain in choline-deficient rats. Experiments similar to those illustrated in Figure 5D–E were conducted in rats subjected to a choline-deficient diet to determine whether choline deficiency can reduce the neuroprotective effects of PNU-120596 in MCAO experiments. (A–B) Typical examples of injured whole-brain coronal sections (2 mm thickness) treated with vehicle (i.e. DMSO) only (A) or PNU-120596 (1 mg·kg^{-1}, i.v., 30 min post-MCAO, n = 4) (B). The results indicate that PNU-120596 remained neuroprotective and significantly reduced MCAO-induced infarct volume (P = 0.0343, n = 4; C) in rats subjected to a choline-deficient diet (unpaired, two-tailed, Student's *t*-test).

mia; and activation of these receptors can enhance neuronal resistance to ischaemic and other types of insults (Meyer *et al.*, 1998; Shimohama *et al.*, 1998; Li *et al.*, 1999; Rosa *et al.*, 2006; Guseva *et al.*, 2008; Quik *et al.*, 2009) as well as cognitive performance in patients and animal models of schizophrenia (Olincy *et al.*, 2006; Olincy and Stevens, 2007), dementia (Kem, 2000; Ren *et al.*, 2007; Bitner *et al.*, 2010) and traumatic brain injury (Guseva *et al.*, 2008). Although choline is a full selective endogenous α7 nAChR agonist, at physiological levels (i.e. <20 μM) and pathophysiological levels expected in the penumbra of brain injury sites (i.e. >20 μM), choline alone is ineffective as an α7 nAChR agonist (Alkondon and Albuquerque, 2006; Djuricic *et al.*, 1991; Klein *et al.*, 1993, 1998; Sarter and Parikh, 2005; Scremin and Jenden, 1989; Uteshev *et al.*, 2003) because of its low potency for α7 activation (EC_{50}~0.5 mM; Papke and Papke, 2002) and

tendency to induce α7 desensitization (IC_{50}~40 μM) (Uteshev *et al.*, 2003). As a result, choline has not previously garnered much attention as a potential neuroprotective agent. However, these limitations may be overcome by the use of PAMs-II, such as previously reported PNU-120596 (Hurst *et al.*, 2005). PNU-120596 inhibits α7 desensitization and thus, can produce a persistent and tunable activation modality of α7 nAChRs (Gusev and Uteshev, 2010; Kalappa *et al.*, 2010), which may be fine-tuned and optimized to achieve neuroprotection (Li *et al.*, 1999; Papke *et al.*, 2000; Uteshev *et al.*, 2003; Uteshev, 2012a,b). Activation of α7 nAChRs can also be achieved by exogenous α7 agonists (e.g. DMXBA; Kem, 2000) or inhibitors of ACh hydrolysis (e.g. donepezil; Kawakami *et al.*, 1996). However, neuroprotection produced by endogenous nicotinic agonists recruited by a PAM-II may be more efficacious and with fewer adverse effects (Lo, 2008; Uteshev, 2012a) because choline is well tolerated and ubiquitously present in the brain, while PNU-120596 (the first PAM-II available on the market) is highly selective for α7 nAChRs and to date, non-α7-mediated effects of PNU-120596 have not been reported. Therefore, this study tested the hypothesis that PNU-120596 enhances survival of neurons subjected to complete oxygen-glucose deprivation (*ex vivo*) and focal cerebral ischaemia (*in vivo*). The rationale for this study was that because activation and desensitization of α7 nAChRs are delicately balanced, the proposed study provided an important proof-of-concept for the development and future clinical trials of PAMs-II, such as PNU-120596 or other functionally related compounds (Faghih *et al.*, 2009; Dinklo *et al.*, 2011; McLean *et al.*, 2011; Freitas *et al.*, 2012; Parada *et al.*, 2013). These factors highlight the main advantage of the PAM-II-based neuroprotective paradigm – the ability of PAMs-II to recruit endogenous choline as a well-tolerated neuroprotective agent ubiquitously present in the extracellular fluid. Although endogenous ambient ACh, along with choline, may also contribute to activation of α7 nAChRs and neuroprotection in the presence of PNU-120596, the extracellular levels of ACh *in vivo* are extremely low (<10 nM) due to ACh hydrolysis (Hartmann *et al.*, 2008) and thus, potential neuroprotective effects of ACh *in vivo* would be expected to arise primarily from synaptic release of ACh. However, PNU-120596 alone did not significantly delay COGD-induced neuronal injury *ex vivo* (Figure 2F) suggesting that at least in acute hippocampal slices, the potential contribution of synaptic ACh to neuroprotection is limited, if any.

The results of *ex vivo* experiments support the need for activation of α7 ion channels as a requirement for α7-mediated enhanced neuroprotection in COGD. This is evidenced by the inhibitory effects of 20 nM MLA on the effects of choline + PNU-120596 (Figure 2C) and the lack of effects by 20 μM choline alone (Figure 2E), 200 μM choline alone (Figure 3) and 1 μM PNU-120596 alone (Figure 2F). By inhibiting α7 nAChR desensitization and enhancing the potency of choline/ACh for α7 activation, PNU-120596 and other PAMs-II allow endogenous choline/ACh to produce a weak persistent level of α7 activation (Gusev and Uteshev, 2010; Kalappa *et al.*, 2010; Uteshev, 2012b) – an activation modality of α7 nAChRs that can be neuroprotective as discussed in this and other studies (Meyer *et al.*, 1998; Shimohama *et al.*, 1998; Li *et al.*, 1999; Rosa *et al.*, 2006). However, the involvement of α7 nAChR-mediated ion channels in the effects of choline + PNU-120596 introduces concerns for neurotoxicity because α7 channels are highly permeable to Ca^{2+} ions (Vernino *et al.*, 1992; Castro and Albuquerque, 1995; Fucile, 2004; Uteshev, 2010) and thus, excessive activation of α7 nAChRs, especially in the presence of PNU-120596, can become toxic for α7-expressing cells, as previously discussed (Li *et al.*, 1999; Del Barrio *et al.*, 2011; Dinklo *et al.*, 2011; Uteshev, 2012a). Despite these valid concerns, our *ex vivo* data demonstrate that neurotoxicity does not result from synergistic action of physiologically relevant 20 μM choline in the presence of 1 μM PNU-120596 (Figure 2A). On the contrary, activation of α7 nAChRs by 20 μM choline + 1 μM PNU-120596 significantly delayed COGD-induced depolarization/injury of CA1 pyramidal neurons *ex vivo* (Figure 2B). These results are further supported by data obtained *in vivo* (Figures 5–7). Moreover, the neuroprotective effects of choline + PNU-120596 are found to be remarkably resilient and somewhat insensitive to the extracellular levels of choline because a 10-fold increase in the choline concentration (i.e. from 20 μM to 200 μM) did not significantly alter the effects of choline + PNU-120596 on injury curves (Figure 3). In addition, the neuroprotective effects of PNU-120596 remained significant in choline-deficient rats (Figure 7). These findings may translate into an enhanced safety and a high therapeutic index of potential PAM-II-based therapies. These properties may allow PAM-II-based therapies to remain effective even under conditions of a highly variable dietary choline intake. Although the molecular mechanisms of neuroprotection by choline + PNU-120596 are currently not known, the Ca^{2+}-dependent and/or -independent JAK2/PI3K/AKT-dependent pathways are likely candidates (Thomsen *et al.*, 2010; Del Barrio *et al.*, 2011) and these and other possible mechanisms will be investigated in our future studies.

The MS analysis of the amounts of PNU-120596 in blood/brain samples demonstrated that PNU-120596 concentrations (sub- to low-μM) achieved and used in our *in vivo* and *ex vivo* studies were comparable and similar to those reported in similar previous studies (McLean *et al.*, 2011). Therefore, it is conceivable that significant neuroprotection after ischaemic insults and the corresponding mechanisms engaged by PNU-120596 in our *ex vivo* and *in vivo* experiments were driven by similar cellular and molecular mechanisms. However, although α7-expressing neurons are the main focus of this study, α7 nAChRs are expressed in a broad variety of non-neuronal tissues including glia (Sharma and Vijayaraghavan, 2001; Parada *et al.*, 2013; Shytle *et al.*, 2004) and immune cells (Wang *et al.*, 2003; De Rosa *et al.*, 2009). Thus, the significant reduction of the infarct volume observed in our *in vivo* experiments may have resulted from a combined net effect of α7 nAChR activation in multiple neuronal and non-neuronal tissues. The impact and contributions of these individual sources of brain protection remain unknown and present a great interest.

The results of our *in vivo* experiments suggest that pre-treatment with PNU-120596 does not produce a true long-term preconditioning: that is, neuroprotection does not remain after PNU-120596 is cleared from the system; because neuroprotection by PNU-120596 was significant only in the 3 h pre-MCAO protocol, but not the 24 h pre-MCAO protocol with PNU-120596 (30 $mg \cdot kg^{-1}$; s.c.) treatments. This is con-

sistent with ~8 h half-life time of PNU-120596 reported previously (McLean *et al.*, 2011). Therefore, the brain does not appear to possess a long-term memory for neuroprotective effects of PNU-120596 and thus, a true preconditioning is ineffective and the presence of PNU-120596 appears to be required for significant neuroprotection.

The chance of neuronal survival after a toxic insult is determined by the neuronal susceptibility to injuries and neuronal sensitivity to therapeutic treatments. In this study, we used an electrophysiological cell injury assay and the COGD protocol to model conditions that are likely present in the core of the ischaemic injury where neuronal depolarization/injury and death can be delayed, but not eliminated (Lo, 2008). The effects of choline + PNU-120596 in hippocampal CA1 pyramidal neurons and CA1 stratum radiatum interneurons then have been compared. While highly effective in CA1 pyramidal neurons, 20 μM choline + 1 μM PNU-120596 failed to delay COGD-induced depolarization/injury of CA1 interneurons (Figure 2D). At least two factors may have contributed to these differences. CA1 interneurons express ~20-fold greater densities of α7 nAChRs than CA1 pyramidal neurons (Kalappa *et al.*, 2010). Thus, concentrations of choline + PNU-120596 that produce only a weak α7 activity in CA1 pyramidal neurons would be expected to produce a ~20-fold greater level of activity in CA1 interneurons. These levels of activation may be excessive and thus, suboptimal and ineffective. However, a 10-fold increase in choline concentration from 20 μM to 200 μM did not produce any harm (Figure 3) suggesting that the beneficial effects of choline + PNU-120596 span over a wide range of choline concentrations and the level of α7 activation may not be the prime reason for the observed differences in the effects of choline + PNU-120596 in CA1 pyramidal and interneurons. Moreover, COGD-induced depolarization/injury of CA1 pyramidal neurons remained significantly delayed by choline + PNU-120596 in the presence of 20 μM GBZ (Figure 4). Therefore, GABAergic neurotransmission in the CA1 pyramidal region does not significantly contribute to neuroprotection by choline + PNU-120596 in COGD *ex vivo*. Another possibility is that CA1 interneurons may be intrinsically more susceptible to acute injury resulted from COGD making choline + PNU-120596 ineffective. This possibility is supported by our data. Indeed, CA1 interneurons are significantly more susceptible to acute injury by COGD than CA1 pyramidal neurons in the presence (Figure 2C) and absence of PNU-120596 (see Results). These results may seem somewhat contradictory to the results reported in some previous studies where hippocampal CA1 interneurons were found less vulnerable to ischaemic insults than hippocampal CA1 pyramidal neurons (Ferrer *et al.*, 1995; Freund and Buzsaki, 1996; Frahm *et al.*, 2004; Avignone *et al.*, 2005; Zhan *et al.*, 2006; Zhan *et al.*, 2007). However, these conclusions were made in relation to delayed (i.e. hours) neuronal injury/death after transient ischaemia, not acute injury/death by COGD investigated in *ex vivo* experiments of this study. Nevertheless, the available data suggest that PAM-II-based neuroprotective protocols may need to be optimized and individualized for specific ischaemic insults, neuronal sub-types and brain subregions reflecting differences in the levels of α7 nAChR expression and the intrinsic neuronal susceptibility to specific ischaemic insults and injuries.

The potential clinical utility of PAMs-II may extend to certain genetic, age- and trauma-related neurodegenerative, sensorimotor and psychiatric disorders characterized by cognitive decline and attention deficits (e.g. schizophrenia, dementia and traumatic brain injury) since these pathologies are also associated with decreased cholinergic tone and a decrease, but not total disappearance, of functional α7 nAChRs (Guan *et al.*, 2000; Olincy *et al.*, 2006; Kelso and Pauly, 2011). Thus, in addition to providing neuroprotective effects, PAMs-II are expected to be able to improve cognitive function and attention impairments in patients and animal models by increasing and partially restoring cholinergic tone (Kem, 2000; Olincy *et al.*, 2006; Ren *et al.*, 2007; Guseva *et al.*, 2008; Bitner *et al.*, 2010; Brown *et al.*, 2010). In this regard, treatments with PNU-120596 or functionally similar compounds may benefit individuals with ischaemic stroke and certain age- and trauma-related cognitive deficits via multiple mechanisms and routes of action.

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Conflict of interest

None.

References

Adams HP Jr, del Zoppo G, Alberts MJ, Bhatt DL, Brass L, Furlan A *et al.* (2007). Guidelines for the early management of adults with ischemic stroke: a guideline from the American Heart Association/American Stroke Association Stroke Council, Clinical Cardiology Council, Cardiovascular Radiology and Intervention Council, and the Atherosclerotic Peripheral Vascular Disease and Quality of Care Outcomes in Research Interdisciplinary Working Groups: the American Academy of Neurology affirms the value of this guideline as an educational tool for neurologists. Stroke 38: 1655–1711.

Alexander SP, Mathie A, Peters JA (2011). Guide to Receptors and Channels (GRAC), 5th edn. Br J Pharmacol 164 (Suppl. 1): S1–S324.

Alkondon M, Albuquerque EX (2006). Subtype-specific inhibition of nicotinic acetylcholine receptors by choline: a regulatory pathway. J Pharmacol Exp Ther 318: 268–275.

Alkondon M, Pereira EF, Cortes WS, Maelicke A, Albuquerque EX (1997). Choline is a selective agonist of alpha7 nicotinic acetylcholine receptors in the rat brain neurons. Eur J Neurosci 9: 2734–2742.

Avignone E, Frenguelli BG, Irving AJ (2005). Differential responses to NMDA receptor activation in rat hippocampal interneurons and pyramidal cells may underlie enhanced pyramidal cell vulnerability. Eur J Neurosci 22: 3077–3090.

Beal CC (2010). Gender and stroke symptoms: a review of the current literature. J Neurosci Nurs 42: 80–87.

Bederson JB, Pitts LH, Germano SM, Nishimura MC, Davis RL, Bartkowski HM (1986). Evaluation of 2,3,5-triphenyltetrazolium chloride as a stain for detection and quantification of experimental cerebral infarction in rats. Stroke 17: 1304–1308.

Bitner RS, Bunnelle WH, Decker MW, Drescher KU, Kohlhaas KL, Markosyan S *et al.* (2010). In vivo pharmacological characterization of a novel selective alpha7 neuronal nicotinic acetylcholine receptor agonist ABT-107: preclinical considerations in Alzheimer's disease. J Pharmacol Exp Ther 334: 875–886.

Breese CR, Adams C, Logel J, Drebing C, Rollins Y, Barnhart M *et al.* (1997). Comparison of the regional expression of nicotinic acetylcholine receptor alpha7 mRNA and [125I]-alpha-bungarotoxin binding in human postmortem brain. J Comp Neurol 387: 385–398.

Brott T, Bogousslavsky J (2000). Treatment of acute ischemic stroke. N Engl J Med 343: 710–722.

Brown KL, Comalli DM, Biasi MD, Woodruff-Pak DS (2010). Trace eyeblink conditioning is impaired in alpha7 but not in beta2 nicotinic acetylcholine receptor knockout mice. Front Behav Neurosci 4: 166.

Castro NG, Albuquerque EX (1995). alpha-Bungarotoxin-sensitive hippocampal nicotinic receptor channel has a high calcium permeability. Biophys J 68: 516–524.

De Rosa MJ, Dionisio L, Agriello E, Bouzat C, Esandi Mdel C (2009). Alpha 7 nicotinic acetylcholine receptor modulates lymphocyte activation. Life Sci 85: 444–449.

Del Barrio L, Martin-de-Saavedra MD, Romero A, Parada E, Egea J, Avila J *et al.* (2011). Neurotoxicity induced by okadaic acid in the human neuroblastoma SH-SY5Y line can be differentially prevented by alpha7 and beta2* nicotinic stimulation. Toxicol Sci 123: 193–205.

Dijkhuizen RM, Beekwilder JP, van der Worp HB, Berkelbach van der Sprenkel JW, Tulleken KA, Nicolay K (1999). Correlation between tissue depolarizations and damage in focal ischemic rat brain. Brain Res 840: 194–205.

Dinklo T, Shaban H, Thuring JW, Lavreysen H, Stevens KE, Zheng L *et al.* (2011). Characterization of 2-[[4-fluoro-3-(trifluoromethyl) phenyl]amino]-4-(4-pyridinyl)-5-thiazoleme thanol (JNJ-1930942), a novel positive allosteric modulator of the {alpha}7 nicotinic acetylcholine receptor. J Pharmacol Exp Ther 336: 560–574.

Djuricic B, Olson SR, Assaf HM, Whittingham TS, Lust WD, Drewes LR (1991). Formation of free choline in brain tissue during in vitro energy deprivation. J Cereb Blood Flow Metab 11: 308–313.

Faghih R, Gopalakrishnan SM, Gronlien JH, Malysz J, Briggs CA, Wetterstrand C *et al.* (2009). Discovery of 4-(5-(4-chlorophenyl)-2-methyl-3-propionyl-1H-pyrrol-1-yl)benzenesulfonami de (A-867744) as a novel positive allosteric modulator of the alpha7 nicotinic acetylcholine receptor. J Med Chem 52: 3377–3384.

Ferrer I, Soriano MA, Vidal A, Planas AM (1995). Survival of parvalbumin-immunoreactive neurons in the gerbil hippocampus following transient forebrain ischemia does not depend on HSP-70 protein induction. Brain Res 692: 41–46.

Frahm C, Haupt C, Witte OW (2004). GABA neurons survive focal ischemic injury. Neuroscience 127: 341–346.

Freitas K, Carroll FI, Damaj MI (2012). The antinociceptive effects of nicotinic receptors alpha7-positive allosteric modulators in murine acute and tonic pain models. J Pharmacol Exp Ther 344: 264–275.

Freund TF, Buzsaki G (1996). Interneurons of the hippocampus. Hippocampus 6: 347–470.

Fucile S (2004). Ca2+ permeability of nicotinic acetylcholine receptors. Cell Calcium 35: 1–8.

Furlan AJ (2002). Acute stroke therapy: beyond i.v. tPA. Cleve Clin J Med 69: 730–734.

Gronlien JH, Hakerud M, Ween H, Thorin-Hagene K, Briggs CA, Gopalakrishnan M *et al.* (2007). Distinct profiles of alpha7 nAChR positive allosteric modulation revealed by structurally diverse chemotypes. Mol Pharmacol 72: 715–724.

Guan ZZ, Zhang X, Ravid R, Nordberg A (2000). Decreased protein levels of nicotinic receptor subunits in the hippocampus and temporal cortex of patients with Alzheimer's disease. J Neurochem 74: 237–243.

Gusev AG, Uteshev VV (2010). Physiological concentrations of choline activate native alpha7-containing nicotinic acetylcholine receptors in the presence of PNU-120596 [1-(5-chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)-urea]. J Pharmacol Exp Ther 332: 588–598.

Guseva MV, Hopkins DM, Scheff SW, Pauly JR (2008). Dietary choline supplementation improves behavioral, histological, and neurochemical outcomes in a rat model of traumatic brain injury. J Neurochem 25: 975–983.

Hartmann J, Kiewert C, Duysen EG, Lockridge O, Klein J (2008). Choline availability and acetylcholine synthesis in the hippocampus of acetylcholinesterase-deficient mice. Neurochem Int 52: 972–978.

Hurst RS, Hajos M, Raggenbass M, Wall TM, Higdon NR, Lawson JA *et al.* (2005). A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization. J Neurosci 25: 4396–4405.

Jin K, Minami M, Lan JQ, Mao XO, Batteur S, Simon RP *et al.* (2001). Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc Natl Acad Sci U S A 98: 4710–4715.

Jorgensen HS, Nakayama H, Raaschou HO, Olsen TS (1999). Stroke. Neurologic and functional recovery the Copenhagen Stroke Study. Phys Med Rehabil Clin N Am 10: 887–906.

Kalappa BI, Gusev AG, Uteshev VV (2010). Activation of functional alpha7-containing nAChRs in hippocampal CA1 pyramidal neurons by physiological levels of choline in the presence of PNU-120596. Plos ONE 5: e13964.

Katzan IL, Furlan AJ, Lloyd LE, Frank JI, Harper DL, Hinchey JA *et al.* (2000). Use of tissue-type plasminogen activator for acute ischemic stroke: the Cleveland area experience. JAMA 283: 1151–1158.

Kawakami Y, Inoue A, Kawai T, Wakita M, Sugimoto H, Hopfinger AJ (1996). The rationale for E2020 as a potent acetylcholinesterase inhibitor. Bioorg Med Chem 4: 1429–1446.

Kelso ML, Pauly JR (2011). Therapeutic targets for neuroprotection and/or enhancement of functional recovery following traumatic brain injury. Prog Mol Biol Transl Sci 98: 85–131.

Kem WR (2000). The brain alpha7 nicotinic receptor may be an important therapeutic target for the treatment of Alzheimer's disease: studies with DMXBA (GTS-21). Behav Brain Res 113: 169–181.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). NC3Rs Reporting Guidelines Working Group. Br J Pharmacol 160: 1577–1579.

Klein J, Holler T, Cappel E, Koppen A, Loffelholz K (1993). Release of choline from rat brain under hypoxia: contribution from phospholipase A2 but not from phospholipase D. Brain Res 630: 337–340.

Klein J, Koppen A, Loffelholz K (1998). Regulation of free choline in rat brain: dietary and pharmacological manipulations. Neurochem Int 32: 479–485.

Li Y, Papke RL, He YJ, Millard WJ, Meyer EM (1999). Characterization of the neuroprotective and toxic effects of alpha7 nicotinic receptor activation in PC12 cells. Brain Res 830: 218–225.

Lo EH (2008). A new penumbra: transitioning from injury into repair after stroke. Nat Med 14: 497–500.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

McLean SL, Idris NF, Grayson B, Gendle DF, Mackie C, Lesage AS *et al*. (2011). PNU-120596, a positive allosteric modulator of alpha7 nicotinic acetylcholine receptors, reverses a sub-chronic phencyclidine-induced cognitive deficit in the attentional set-shifting task in female rats. J Psychopharmacol 26: 1265–1270.

Meyer EM, Tay ET, Zoltewicz JA, Papke RL, Meyers C, King M *et al*. (1998). Neuroprotective and memory-related actions of novel alpha-7 nicotinic agents with different mixed agonist/antagonist properties. J Pharmacol Exp Ther 284: 1026–1032.

Olincy A, Stevens KE (2007). Treating schizophrenia symptoms with an alpha7 nicotinic agonist, from mice to men. Biochem Pharmacol 74: 1192–1201.

Olincy A, Harris JG, Johnson LL, Pender V, Kongs S, Allensworth D *et al*. (2006). Proof-of-concept trial of an alpha7 nicotinic agonist in schizophrenia. Arch Gen Psychiatry 63: 630–638.

Papke RL, Papke JKP (2002). Comparative pharmacology of rat and human alpha7 nAChR conducted with net charge analysis. Br J Pharmacol 137: 49–61.

Papke RL, Bencherif M, Lippiello P (1996). An evaluation of neuronal nicotinic acetylcholine receptor activation by quaternary nitrogen compounds indicates that choline is selective for the alpha 7 subtype. Neurosci Lett 213: 201–204.

Papke RL, Meyer E, Nutter T, Uteshev VV (2000). alpha 7 receptor-selective agonists and modes of alpha 7 receptor activation. Eur J Pharmacol 393: 179–195.

Parada E, Egea J, Buendia I, Negredo P, Cunha AC, Cardoso S *et al*. (2013). The microglial alpha7-acetylcholine nicotinic receptor is a key element in promoting neuroprotection by inducing heme oxygenase-1 via nuclear factor erythroid-2-related factor 2. Antioxid Redox Signal, in press.

Quik M, Huang LZ, Parameswaran N, Bordia T, Campos C, Perez XA (2009). Multiple roles for nicotine in Parkinson's disease. Biochem Pharmacol 78: 677–685.

Ren K, Thinschmidt J, Liu J, Ai L, Papke RL, King MA *et al*. (2007). alpha7 Nicotinic receptor gene delivery into mouse hippocampal neurons leads to functional receptor expression, improved spatial memory-related performance, and tau hyperphosphorylation. Neuroscience 145: 314–322.

Richard Green A, Odergren T, Ashwood T (2003). Animal models of stroke: do they have value for discovering neuroprotective agents? Trends Pharmacol Sci 24: 402–408.

Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB *et al*. (2012). Heart disease and stroke statistics – 2012 update: a report from the American Heart Association. Circulation 125: e2–e220.

Rosa AO, Egea J, Gandia L, Lopez MG, Garcia AG (2006). Neuroprotection by nicotine in hippocampal slices subjected to oxygen-glucose deprivation: involvement of the alpha7 nAChR subtype. J Mol Neurosci 30: 61–62.

Rossi DJ, Oshima T, Attwell D (2000). Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature 403: 316–321.

Sarter M, Parikh V (2005). Choline transporters, cholinergic transmission and cognition. Nat Rev Neurosci 6: 48–56.

Scremin OU, Jenden DJ (1989). Focal ischemia enhances choline output and decreases acetylcholine output from rat cerebral cortex. Stroke 20: 92–95.

Sharma G, Vijayaraghavan S (2001). Nicotinic cholinergic signaling in hippocampal astrocytes involves calcium-induced calcium release from intracellular stores. Proc Natl Acad Sci U S A 98: 4148–4153.

Shimohama S, Greenwald DL, Shafron DH, Akaike A, Maeda T, Kaneko S *et al*. (1998). Nicotinic alpha 7 receptors protect against glutamate neurotoxicity and neuronal ischemic damage. Brain Res 779: 359–363.

Shytle RD, Mori T, Townsend K, Vendrame M, Sun N, Zeng J *et al*. (2004). Cholinergic modulation of microglial activation by alpha 7 nicotinic receptors. J Neurochem 89: 337–343.

Sitzia F, Brown JT, Randall AD, Dunlop J (2011). Voltage- and temperature-dependent allosteric modulation of alpha7 nicotinic receptors by PNU-120596. Front Pharmacol 2: 81.

Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990). A semiautomated method for measuring brain infarct volume. J Cereb Blood Flow Metab 10: 290–293.

Tanaka E, Yamamoto S, Kudo Y, Mihara S, Higashi H (1997). Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro. J Neurophysiol 78: 891–902.

Thomsen MS, Hansen HH, Timmerman DB, Mikkelsen JD (2010). Cognitive improvement by activation of alpha7 nicotinic acetylcholine receptors: from animal models to human pathophysiology. Curr Pharm Des 16: 323–343.

Uteshev VV (2010). Evaluation of Ca2+ permeability of nicotinic acetylcholine receptors in hypothalamic histaminergic neurons. Acta Biochim Biophys Sin (Shanghai) 42: 8–20.

Uteshev VV (2012a). alpha7 nicotinic ACh receptors as a ligand-gated source of Ca(2+) ions: the search for a Ca (2+) optimum. Adv Exp Med Biol 740: 603–638.

Uteshev VV (2012b). Somatic integration of single ion channel responses of alpha7 nicotinic acetylcholine receptors enhanced by PNU-120596. Plos ONE 7: e32951.

Uteshev VV, Meyer EM, Papke RL (2003). Regulation of neuronal function by choline and 4OH-GTS-21 through alpha 7 nicotinic receptors. J Neurophysiol 89: 1797–1806.

Vernino S, Amador M, Luetje CW, Patrick J, Dani JA (1992). Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. Neuron 8: 127–134.

Wang H, Yu M, Ochani M, Amella CA, Tanovic M, Susarla S *et al*. (2003). Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation. Nature 421: 384–388.

Whiteaker P, Davies AR, Marks MJ, Blagbrough IS, Potter BV, Wolstenholme AJ *et al*. (1999). An autoradiographic study of the distribution of binding sites for the novel alpha7-selective nicotinic radioligand [3H]-methyllycaconitine in the mouse brain. Eur J Neurosci 11: 2689–2696.

Woodruff TM, Thundyil J, Tang SC, Sobey CG, Taylor SM, Arumugam TV (2011). Pathophysiology, treatment, and animal and cellular models of human ischemic stroke. Mol Neurodegener 6: 11.

Young GT, Zwart R, Walker AS, Sher E, Millar NS (2008). Potentiation of alpha7 nicotinic acetylcholine receptors via an allosteric transmembrane site. Proc Natl Acad Sci U S A 105: 14686–14691.

Zhan RZ, Nadler JV, Schwartz-Bloom RD (2006). Depressed responses to applied and synaptically-released GABA in CA1 pyramidal cells, but not in CA1 interneurons, after transient forebrain ischemia. J Cereb Blood Flow Metab 26: 112–124.

Zhan RZ, Nadler JV, Schwartz-Bloom RD (2007). Impaired firing and sodium channel function in CA1 hippocampal interneurons after transient cerebral ischemia. J Cereb Blood Flow Metab 27: 1444–1452.